Transient injury to rat lung mitochondrial DNA after exposure to hyperoxia and inhaled nitric oxide

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Transient injury to rat lung mitochondrial DNA after exposure to hyperoxia and inhaled nitric oxide. Am J Physiol Lung Cell Mol Physiol 286: L23–L29, 2004. First published February 7, 2003; 10.1152/ajplung.00352.2002.—The effect of hyperoxia alone and in combination with inhaled nitric oxide (NO) on the integrity of lung mitochondrial DNA (mtDNA) in vivo was evaluated in Fischer 344 rats. PCR amplification of lung mtDNA using two sets of primers spanning 10.1 kb of the mtDNA revealed that inhalation of 20 ppm of NO in conjunction with hyperoxia (>95% O2) reduced the amplification of mtDNA templates by 10 ± 1% and 26 ± 3% after 24 h of exposure. The ability of mtDNA to amplify was not compromised in rats exposed to 80% O2, even in the presence of 20 ppm of inhaled NO. Surprisingly, exposure to >95% O2 alone for either 24 or 48 h did not compromise the integrity of mtDNA templates compared with air-exposed controls, despite evidence of genomic DNA injury. Interestingly, inhaling NO alone for 48 h increased mtDNA amplification by 12 ± 2% to 21 ± 7%. Injury to the lung mtDNA after exposure to >95% O2 plus 20 ppm of NO was transient as rats allowed to recover in room air after exposure displayed increased amplification, with levels exceeding controls by 20 ± 3% to 29 ± 4%. Increased amplification was not due to cellular proliferation or increased mitochondrial number. Moreover, the ratio of pulmonary mtDNA to genomic DNA remained the same between treatment groups. The results indicate that hyperoxia fails to induce significant injury to mtDNA, whereas inhalation of NO with hyperoxia results in mtDNA damage, the lesions are rapidly repaired during recovery.

Oxidative stress; acute lung injury; deoxyribonucleic acid

MITOCHONDRIAL DNA (mtDNA) is thought to be particularly susceptible to damage by reactive species; it is located in close proximity to the electron transport chain that has been considered a major source of reactive species, and it is not complexed with DNA binding proteins, such as histones, and other components of the protein scaffold, which are normally associated with nuclear DNA (5, 20, 30, 34, 37). Although reactive species generation is generally considered to be a normal consequence of aerobic metabolism, increases in rates of reactive species production may overwhelm cellular antioxidant capacity and lead to the oxidation of macromolecules within both the cytoplasm and cellular organelles. Increase in the rate and magnitude of reactive oxygen species generation by mitochondria has been best documented during hyperoxic lung injury (16, 44, 45). Exposure to high levels of O2 (>95%) has been shown to result in increased oxidative damage to a wide range of cellular macromolecules, including proteins, lipids, and nucleic acids, within the first 48 h of continuous exposure (16, 23, 27). The oxidation of macromolecules advances to cellular injury and death of vascular and pulmonary cells, resulting in pulmonary edema and eventually death at roughly 60 h (for adult Fischer 344 rats) under continuous exposure (16). Therefore, exposure to hyperoxia, with the well-documented increased production of reactive species by mitochondria, may represent a suitable model to investigate oxidative injury to the mtDNA. Documentation of mtDNA injury in vivo after acute insult is sparse compared with the number of elegant in vitro and cell model systems that have demonstrated oxidative injury to mtDNA (2, 12, 13, 20, 40, 47). Animal model systems of acute injury that have evaluated oxidative mtDNA damage include cigarette smoke-exposed cardiovascular tissue (1, 25), ischemia-damaged cardiac and neural tissues (9, 22), and during alterations in iron homeostasis (46).

Inhalation of high concentrations of O2 in combination with inhaled nitric oxide (NO) has recently been utilized as a treatment of pulmonary hypertension, acute lung injury, and complications stemming from premature birth (3, 10, 28). The clinical value of inhaled NO for the treatment of pulmonary hypertension lies primarily in its ability to cause vascular dilation, resulting in increased blood flow and improved oxygenation (3, 10). From a therapeutic standpoint, these effects of inhaled NO (20 ppm of inhaled NO is presently used at most clinical settings) allow the levels of inhaled O2 to be reduced in many cases, potentially minimizing the deleterious effects of administration of high O2 for extended periods. In animal models of hyperoxia coadministration of inhaled NO at levels <20 ppm increased the survival time to hyperoxia and reduced pulmonary vascular leak and inflammatory activation (19, 21). Inhalation of NO at 80 ppm with hyperoxia has been found to aggravate hyperoxic lung injury (29, 35). NO has been shown to possess a number of chemical reactive pathways and to regulate mitochondrial respiration, including the production of reactive intermediates by the electron transport chain, which protects cells and tissues from oxidative insults (19, 32, 33, 38). However, NO-derived reactive nitrogen species, such as nitrogen dioxide and peroxynitrite, are potentially destructive and modify macromolecules, causing cellular injury and death.

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(6, 14, 23, 25). For example, increased nitration of proteins in lung airway, alveolar epithelium, and vascular cells has been observed in hyperoxic models of acute lung injury (23, 27, 29). Whereas several studies have investigated the effects of NO on mitochondrial functions (4, 6, 18, 32), the effect on mtDNA is virtually unknown.

The current study was designed to evaluate the injury to lung mtDNA after both lethal (>95%) and sublethal (80%) hyperoxic exposures, which represent well-characterized animal models of acute lung injury that include mitochondrial-driven production of reactive species. Injury to the pulmonary mitochondrial genome was also evaluated after exposure to both hyperoxic conditions plus 20 ppm of inhaled NO. Because the mitochondrial possesses unique DNA repair systems that maintain the integrity of the mitochondrial genome (5, 26, 37, 43), the persistence of mtDNA injury was evaluated 24 and 48 h after the animals were removed from exposure to hyperoxia plus NO.

METHODS

Animals. Male Fischer 344 rats (175–200 g) were obtained from Charles River Laboratories, had access to food and water ad libitum, were maintained on a 12-h light/dark cycle, and were housed in the institutional animal facility. All protocols were carried out under the institutional animal care and use committee regulations at Children’s Hospital of Philadelphia.

Inhaled gas treatment and sample collection. Animals in standard rat cages were placed in a polypropylene exposure chamber, and mixtures of O2, NO, and air (BOC Gases, Bellmawr, NJ) were introduced at a rate of 5 liters/min. O2 and air (>95% O2, 80% O2/20% N2, and breathable air) flow rates were controlled by calibrated regulators. NO flow into the carrier gas stream was controlled by an INOvent apparatus (INO Therapeutics, Clinton, NJ). NO levels (20 ppm) were continually monitored and modulated in the gas stream introduced into the treatment chamber. Nitrogen dioxide levels in the treatment gas were maintained at 0.1–0.2 ppm. Exhaust gases from the treatment chamber were vented into a chemical fume hood. Treatment duration was 24 or 48 h, with some groups receiving a recovery period of 24 or 48 h in room air after cessation of gas treatment. These treatment conditions did not result in the mortality of any animals in any of the treatment groups. After treatment, the rats were removed from the chamber and anesthetized with pentobarbital sodium (50 mg/kg) injected intraperitoneally. Animals were ventilated with room air via a tracheotomy tube inserted into an incision in the trachea, and ventilation was maintained at a rate of 50 bpm with a tidal volume of 4 ml. After exposure of the thoracic cavity, lungs were cleared of blood with room temperature PBS via the pulmonary artery.

Immunohistochemistry. Paraffin-embedded PFA-fixed lung tissue was sectioned and subjected to immunohistochemistry. Paraffin-embedded PFA-fixed lung tissue was sectioned and subjected to immunohistochemistry. The right lobe of the lung was ligated, removed, snap-frozen in liquid nitrogen, and stored at −80°C until being processed for DNA preparation. The remainder of the organ was inflated via the trachea with 4% paraformaldehyde (PFA) in PBS, pH 7.2, followed by overnight immersion in 4% PFA. Lungs were stored in 70% ethanol until paraffin embedding and sectioning for immunohistochemistry.

DNA preparation. Frozen lung tissue was processed to a fine powder in a low-temperature homogenizer to maintain it in a frozen state and to prevent activation of endogenous nucleases. Approximately 1 g of frozen tissue was homogenized and mixed three times by agitating the powder to ensure thorough mixing and randomization of different areas of the lung for use in subsequent assays. Aliquots of each sample were stored at −80°C until total cellular DNA was prepared. Extraction protocols were those of the manufacturer, except samples were heated to 50°C and vortexing was avoided in favor of gentle mixing to prevent thermal and shear stress damage to the DNA. After elution from the QIAamp DNA mini-kit (Qiagen, Valencia, CA), DNA was dialyzed against sterile, distilled water overnight, quantified by the PicoGreen assay (Molecular Probes, Eugene, OR), and stored as single-use aliquots at −20°C to prevent freeze-thaw artifacts. DNA samples used as templates for quantitative PCR were quantified in triplicate; triplicate samples that were not within 5% of each other were discarded, and new samples were prepared and quantified.

PCR linearity assay. To ensure that the degree of amplification of the mtDNA was solely a function of the amount of template present, amounts of 0, 5, 10, and 20 ng of rat lung total cellular DNA from untreated animals were prepared and subjected to PCR using primer set 2 as described below.

mtDNA oxidation with methylene blue and light. Rat lung total DNA from control animals was mixed with methylene blue (2 μM) and exposed to light for 0, 1, 2, 3, or 15 min as described in detail previously (15). Control removals were exposed to light in the absence of methylene blue. After removal of methylene blue and purification of the DNA, PCR was performed using primer set 2 as described below.

PCR template integrity assay. The PCR assay was performed using a PE 9600 thermal cycler (ABI/Perkin Elmer, Foster City, CA) in a total volume of 25 μl with the following buffer: 1× XL buffer, 0.75 mM MgOAc, 0.2 mM each dNTP, and 25 pmol each primer. Primers for PCR were 1 TTT TGA CAC CTG GCC ATG GAC AA-3′ and 5′-GTT GGT TGT AGG GCT AGG TAG-3′; primer set 1 (nt 5430–10340) 5′-TAG GAC AGC CAG GAC CCC TCC-3′ and 5′-TGA GTG GGG TGG ATA ATG GAT CCC-3′. Thermal cycling was performed as follows: hot start addition of 1 unit of Tth polymerase (Perkin Elmer) at 80°C, then 18 cycles of 94°C for 15 s and 61°C for 6 min, followed by a final extension of 10 min at 72°C. Amounts of amplified DNA product were quantified using the PicoGreen assay, with each reaction assayed in triplicate. Triplicates that did not test to within 5% of each other were discarded, and the PCR reaction was re-assayed. Three DNA preparations from each of three animals in each treatment group were assayed twice for mtDNA amplification using both primer sets, resulting in a total of 36 mtDNA-specific amplification reactions per group. Each set of PCR reactions was standardized using a rat lung DNA preparation prepared as above to correct for interassay variation.

Southern blotting. Total cellular DNA samples prepared as above were digested to completion with KpnI (New England Biolabs, Beverly, MA), run on 0.7% agarose gels, and blotted to nylon membranes (Amersham, Buckinghamshire, UK) by alkaline capillary transfer using standard procedures. Hybridization probes were prepared by PCR using standard methods and the following primers: mtDNA primer set 1 (nt 1114–12130) 5′-GAC AGC CAG GAC CCC TCC-3′ and 5′-TGA GTG GGG TGG ATA ATG GAT CCC-3′. 32P-labeled probes were prepared by random priming and hybridized overnight to membranes in a buffer containing 5× SSC, 5× Denhardt’s solution, 1% SDS, and 100 μg/ml denatured salmon sperm DNA, followed by washing at 65°C in 0.2× SSC and 0.1% SDS. Signals were visualized by autoradiography with Kodak BioMax film and quantified by densitometry with MacBas (Fuji Medical Systems, Stamford, CT). DNA from each of three animals from each treatment group was assayed twice by this method.

Data analysis. Differences in the amount of mtDNA amplification from each gas inhalation group were examined using the Kruskal-Wallis ANOVA on ranks test from SigmaStat v2.0 (Jandel, San Rafael, CA). Differences in the ratio of amounts of genomic DNA and mtDNA between groups were assessed using Tukey’s test for multiple comparisons.

Immunohistochemistry. Paraffin-embedded PFA-fixed tissues were cut in 5-μm sections and affixed to glass microscope slides (Superfrost; Menzer). Paraffin was removed by 10 min in xylene and rehydration by standard methods, an antigen retrieval procedure was performed. Sections were treated at 100°C for 10 min in 0.1 M citrate buffer, pH 6.5, followed by removal of the boiling vessel from the hot.
plate and allowing the slides to remain in the hot buffer for an additional 10 min. Sections were then treated with 1% sodium borohydride for 3 × 10 min to reduce endogenous tissue fluorescence. Tissues were blocked with 10% normal goat serum in PBS, pH 7.2/0.1% Tween 20, for 1 h at room temperature, incubated overnight at 4°C with anti-Ki67 antibody (BD Biosciences, San Diego, CA) diluted 1:1,000 in blocking buffer, washed three times with PBS/Tween, and incubated for 1 h with Alexafluor-conjugated anti-mouse IgG antibody (Molecular Probes) diluted 1:1,000 in PBS/Tween. Sections were examined for secondary antibody binding with an excitation wavelength of 480 nm and an emission wavelength of 519 nm.

RESULTS

PCR linearity assay. Quantitative PCR, which has been utilized previously to determine injury to mtDNA in cell models and in vivo (1, 2, 47), was employed to assess the injury to mtDNA in rat lungs. To ensure that the degree of mtDNA template amplification was solely a function of the amount of intact template in the reaction, 0–20 ng of rat lung total DNA were subjected to PCR using two sets of primers, with each set resulting in the generation of a single PCR product (Fig. 1A). Data in Fig. 1B also show that the PCR conditions used in these experiments result in a linear increase in PCR product concentration as a function of the input rat lung DNA template added to the reaction. On the basis of these results, 10 ng of DNA were used in the PCR template integrity assays. Under the amplification procedures utilized, primer 2 (nt 5430–10340) consistently resulted in higher product yield (Fig. 1A), likely reflecting differences in template secondary structure, nucleotide sequence, or unequal rate of primer/template hybridization during PCR. Separation of the extracted mtDNA, used as a template for the PCR assay, in agarose gels (16) failed to show any differences in the ratio of intact supercoiled mtDNA to nicked or linearized molecules between the control and treated animals (data not shown). Moreover, data in Fig. 1C indicate that the methodology employed was able to detect injury to the mtDNA. Total lung DNA preparations exposed to light in the presence of methylene blue, which has been shown to generate 8-hydroxyguanine and other oxidative lesions to DNA (15), showed a reduction in amplification that was dependent on the length of exposure. These results indicate that the yield of specific mtDNA amplification product is a function of the degree of damage to the mtDNA template.

mtDNA template integrity assessment. The amount of mtDNA-derived product resulting from the PCR amplification of total rat lung DNA from each of the gas inhalation treatment groups was quantified and compared with amplification from control (room air) animals. The results represent the average of three different mtDNA preparations from each of three animals from every treatment group assayed twice for amplification using both primer sets. Exposure to >95% O2 for either 24 or 48 h alone did not cause any significant damage to mtDNA (Fig. 2). Rats treated with 20 ppm of NO alone displayed slightly higher levels of mtDNA amplification than controls, with the 48-h group showing significantly higher amplification using primer set A. A significant decrease in the ability of the input mtDNA templates to amplify was observed when hyperoxia was combined with 20 ppm of NO. A reduction in amplification of 10 ± 1% for primer 1 (Fig. 2A) and 26 ± 3% (average ± SE) for primer 2 (Fig. 2B) was observed at 24 h of exposure to the two gases. Similarly, a reduction in amplification of 7 ± 2% and 23 ± 3% for primers 1 and 2, respectively, was observed in the 48-h treatment group (Fig. 2). The reduction in amplification after 48 h corresponds to a 0.10 ± 0.01 (n = 18) and 0.35 ± 0.04 (n = 17) lesion frequency per 5 kb of mtDNA for primers 1 and 2, respectively, using the DNA lesion frequency calculation described previously (47).

In contrast to the injurious effect of respiring NO in the presence of >95% O2, inhalation of 20 ppm of NO in the presence of 80% O2 failed to induce any decline in the amplification of mtDNA (Fig. 3). Inhalation of 80% O2 alone did not cause any damage to mtDNA that significantly reduced its amplification by PCR (Fig. 3).

To examine whether the lesioning of the mtDNA that prevents its ability to amplify persists after cessation of the exposure, animals subjected to >95% O2 plus 20 ppm of NO for 24 h were allowed to recover for 24 or 48 h in room air. The

![Fig. 1](http://ajplung.physiology.org/)
data in Fig. 4 indicate that mtDNA input templates from rat lungs that were allowed to recover for either 24 or 48 h displayed significantly higher levels of mtDNA amplification compared with controls and with animals exposed to >95% O₂ plus 20 ppm of NO for 24 h (Fig. 4). mtDNA extracted from rat lungs after 24 h of recovery showed 18 ± 2% and 19 ± 3% (for primers 1 and 2, respectively) increases in amplification compared with controls. The increased amplification was sustained even after an additional 24-h recovery in room air, as indicated by the 20 ± 3% and 29 ± 4% (for primers 1 and 2, respectively) increases in amplification 48 h after cessation of treatment.

Mitochondrial/genomic DNA ratio. Southern hybridizations were performed to determine whether the observed differences in mtDNA amplification were due to different amounts of mtDNA present in the total cellular DNA isolated from the animals in the different treatment groups. Genomic DNA was probed with a sequence from the rat 18S ribosomal DNA, and mtDNA was probed with a DNA sequence derived from nt...
whereas hyperoxia alone may result in the increased generation of reactive oxygen and nitrogen species, the addition of NO facilitates the production of reactive nitrogen species, which contribute to chemical modification of a wide array of cellular macromolecules, such as proteins, carbohydrates, lipids, and nucleic acids (17, 23, 27, 29). This study addresses the effects of oxygen and NO inhalation on the integrity of mtDNA and the persistence of damaged mtDNA after the cessation of inhalation treatment.

Hyperoxia is a model of acute lung injury characterized by mitochondrial production of reactive intermediates as well as mitochondrial dysfunction that precedes cell death and gross morphological pulmonary injury (16, 44, 45). Despite the well-documented production of reactive oxygen species within the mitochondria, the data in Fig. 3 revealed that exposure to hyperoxia for up to 48 h (a time of documented mitochondrial production of reactive intermediates) failed to generate significant lesioning of the mtDNA that would prevent its amplification. Although it is known that reactive species damage mtDNA in cell models (2, 12, 47), there is no record of mtDNA damage associated with inhaled O2 treatment in vivo. Failing to find significant differences in mtDNA amplification between control and hyperoxic gas inhalation groups may indicate an efficient removal of reactive species by mitochondrial antioxidant systems, such as mitochondrial superoxide dismutase, or that the reactivity of the oxidants generated within the mitochondria is diverted to other mitochondrial targets away from mtDNA. Alternatively, mtDNA repair systems may be coordinately upregulated during hyperoxia and inhalation of NO, and as such, no net gain in lesion frequency occurs.

The increase in damage observed in the >95% O2/20 ppm of NO groups may reflect the increased formation of reactive oxygen and nitrogen species, such as peroxynitrite, the reaction of reactive oxygen and nitrogen species, such as peroxynitrite, with NO groups may re...
product of NO and superoxide, and nitrogen dioxide that have been described both in vitro and in cell models to cause lesions in DNA (8, 14, 39, 42, 48). Reactive oxygen and nitrogen species have been found to produce DNA base lesions such as 8-nitroguanine, 8-oxoguanine, oxazolone, and 4,5-dihydro-5-hydroxy-4-nitrosoxy-2′-deoxyguanosine (8, 14, 39, 48) in a sequence-dependent manner that block DNA polymerase progression in quantitative PCR assays for DNA damage (36, 37). The sequence specificity in mtDNA lesion prevalence may explain the difference in the amount of damage detected by the two discrete, nonoverlapping primer sets (Fig. 2), reflecting the previously recognized differing susceptibility of mtDNA regions to injury (12, 36, 37, 47). The frequency of mtDNA lesions after 48 h of exposure to 95% O2 plus 20 ppm of NO, calculated as described before (47), is similar to mtDNA lesion frequency found after exposure of cells to 100 μM H2O2 (47) or after exposure of human smooth muscle or human endothelial cells to 100 μM peroxynitrite or to peroxynitrite generators (2).

The data presented here indicate that there is a small but significant increase in mtDNA damage when 20 ppm of NO is administered in conjunction with >95% O2, but this damage dissipates within 24 h after cessation of treatment. The restoration of mtDNA integrity after injury likely reflects the presence of repair systems. It is now recognized that mtDNA-specific repair systems are induced in response to oxidative insults to restore the fidelity of the injured mitochondrial genome (5, 9, 11–13, 26, 33).

NO may play a critical role in the induction of mtDNA repair systems. Although mtDNA may be continuously modified by oxidants, the lesions are rapidly and efficiently repaired (5, 37). The significant increase in amplification noted in Fig. 2 with primer 2 in animals exposed to NO alone for 48 h may be the result of an efficient repair of mtDNA. Recent reports indicate that low levels of NO can reduce the amount of mtDNA damage caused by high-level NO exposure in keratinocytes (38) as well as prevent cellular DNA damage from other reactive species, such as hydrogen peroxide, in cultured fibroblasts (33). The data presented herein indicate that any damage caused by the inhaled gas mixtures not only disappears by 24 h but also that the mtDNA in the lung displays a lower level of damage after the injury-stimulated repair processes, as has been previously noted in cell models (33). The increased integrity of the mitochondrial genome after treatment with NO alone as well as in those animals allowed to recover from the most damaging O2 plus NO treatment was surprising and likely due to a NO-dependent augmentation of mtDNA repair mechanisms. The increased amplification efficiency of mtDNA by either NO inhalation under normoxic conditions or during the recovery period after exposure to >95% O2 plus NO is not due to cellular proliferation or increased mtDNA content (7, 24). Although control of mitochondrial proliferation and mtDNA replication in the absence of cell division, particularly as it relates to tissue damage and repair is not well understood at this time (31), the results indicate that there was no significant amount of mtDNA replication in the lung as a consequence of experimental treatment (Fig. 5).

Improved oxygenation that ultimately lowers the need for inspiring high oxygen concentrations and thus minimizing the effects of long-term hyperoxia on the pulmonary system is a major therapeutic benefit of inhaled NO. The data suggest that inhaled NO may also facilitate the rapid and efficient repair of injured mtDNA, indicating another potential clinical benefit of administering low levels of inhaled NO.

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REFERENCES

What regulates mitochondrial DNA copy number in animal disease, oxidative stress, and senescence.


